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Arginase is a major pathway of L-arginine metabolism in nephritic glomeruli

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Arginase is a major pathway of L-arginine metabolism in nephritic glomeruli. L-arginine can be metabolized to nitric oxide (NO) by nitric oxide synthase (NOS) and to urea and L-ornithine by arginase. Competition between these pathways for L-arginine in inflammatory sites has been suggested. In experimental glomerulonephritis glomeruli produce nitrite; a major source is macrophages. We hypothesized that arginase is present in glomeruli and may compete for substrate with NOS in glomerulonephritis. Therefore we examined both pathways in isolated nephritic glomeruli and peritoneal macrophages. Arginase activity was present in glomeruli, increased by >500% in nephritic glomeruli compared to controls, and was predominant over NOS. Activity increased with L-NMMA (a NOS inhibitor), but this trend did not reach statistical significance. In macrophages both pathways were present; NOS predominated basally but this was reversed by L-NMMA. In contrast with glomeruli macrophage arginase activity increased after LPS stimulation. Levels of macrophage arginase activity could not account for activity in nephritic glomeruli, suggesting another source of arginase. This is the first demonstration of high arginase activity of nephritic glomeruli. Competition between arginase and NOS pathways suggests a regulatory mechanism of L-arginine metabolism within the glomerulus, with implications for the pathogenesis of injury and scarring in glomerulonephritis.

L-arginine is the substrate for nitric oxide synthesis in mammalian cells and is also metabolized to L-ornithine and urea by the action of arginase. We have recently reported that glomeruli in experimental glomerulonephritis synthesize nitrite (NO_2^-) from L-arginine via nitric oxide [1, 2], and have produced evidence that the major source is macrophages infiltrating the glomerulus [3]. Activated macrophages accumulate and are responsible for glomerular injury in the nephrotoxic model, but the mechanisms of injury are still unknown.

As macrophages can express both NOS and arginase activities [4–6], we investigated the hypothesis that the arginase pathway is also present in the glomerulus and may compete for substrate with NOS in glomerulonephritis.

Recently Albina et al [7] have examined the temporal expression of different pathways of L-arginine metabolism in healing wounds in the rat, and showed that early in the course of wound healing levels of nitric oxide synthase are high, whereas later there is high arginase activity and arginine levels in wound fluids are low. Currie, Gyure and Cifuentes [8] suggested that arginase activity may compete for L-arginine in the microenvironment of an inflammatory site. Hibbs et al [9, 10] have attributed the cytotoxic effects of activated macrophages to nitric oxide. Arginase however has been reported to inhibit tumor cell growth [11], and a strong relation between increased arginase activity and allograft rejection has been reported [12].

We studied both pathways of L-arginine metabolism in isolated glomeruli in nephrotoxic nephritis under basal conditions and with exogenous stimuli known to activate or inhibit inducible NOS. We also studied these events in isolated macrophages, as these cells form a major component of the inflammatory reaction in nephrotoxic nephritis.

Methods

Animals

Inbred male Lewis rats (St. Mary's Hospital Medical School) were used for all experiments.

Induction of glomerulonephritis

Accelerated nephrotoxic nephritis was induced as previously described [1]. Both nephritic and rabbit globulin (RG) immunized control rats were fed a nitrite and nitrate free diet from three days before preimmunization until the end of the experiment as part of a study of urinary nitrate excretion to be reported elsewhere. Rats were immunized with 1 mg rabbit immunoglobulin (Sigma) in Freunds complete adjuvant (Sigma) i.p. seven days before an i.v. subnephritogenic dose of rabbit anti-rat nephrotoxic globulin (nephritic rats) or control rabbit globulin (RG immunized control rats). Urine was collected from rats placed in metabolic cages. Proteinuria was measured by sulphosalicylic acid method.

Glomerular isolation

Glomeruli were isolated from control or nephritic kidneys perfused in vivo with 50 ml sterile pyogen free saline (Phoenix Pharmaceuticals, Gloucester, UK) at room temperature as previously described [13] three days after i.v. globulin. They were washed twice and plated at 2000/ml in 16 mm plastic tissue culture wells (Nunc, Uxbridge, UK) and incubated for 48 hours at 37°C under 4% CO_2. Culture medium was DMEM without phenol red supplemented with 10% FCS (Flow laboratories); glutamine (Sigma) 584 mg/liter; and penicillin 100 U/ml, streptomycin 100 μg/ml and amphotericin B 250 ng/ml (Sigma). The

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concentration of L-arginine in this medium is 0.4 mM. The FCS contained 0.12 ng/ml endotoxin as determined by the supplier. L-[Guanido-^{14}C]-arginine (NEN, Cambridge, Massachusetts, USA) (0.25 μM) was added to medium to provide approximately 2×10^5 cpm/ml. Medium in some wells was supplemented with LPS (*E. coli* 0127:B8, Sigma) 1 μg/ml or 100 μg/ml, or with 300 μM N^G-monomethyl-L-arginine (L-NMMA, gift of Dr. S. Moncada, Wellcome Research Laboratories, Beckenham, Kent, UK).

Isolation of peritoneal macrophages

Peritoneal macrophages were obtained by peritoneal lavage with calcium free perfusion buffer (pH 7.4) from rats injected six days previously i.p. with 10 ml thioglycollate broth (Oxoid) or rats injected 10 days previously with 0.2 ml Corynebacterium parvum (7 mg/ml) (Wellcome). Macrophages were plated at 10^6/ml in 16 mm wells and after two hours of incubation were washed three times to remove non-adherent cells. They were cultured in DMEM without phenol red supplemented with 10% FCS, glutamine 584 mg/liter, and penicillin 100 U/ml, streptomycin 100 μg/ml and amphotericin B 250 ng/ml (normal medium) for 48 hours at 37°C under 4% CO_2. Medium in some wells was supplemented with L-[Guanido-^{14}C]-arginine to provide approximately 2×10^5 cpm/ml for extracellular arginase activity assay. In experiment 1 thioglycollate macrophages were studied for NO_2^-, intra- and extracellular arginase activity. Medium in some wells were supplemented with rat gamma-interferon 100 U/ml (IFN, Holland Biotechnology BV, Leiden, The Netherlands) or with LPS 1 μg/ml or 100 μg/ml, or with 300 μM L-NMMA. In experiment 2 thioglycollate and C. parvum macrophages were studied. In some of these experiments L-arginine free DMEM with FCS and antibiotics as above was used to determine the effects of low L-arginine concentrations on NO_2^- production and viability.

Nitrite assay

Nitrite (NO_2^-) in culture supernatants was measured at 48 hours by the Griess reaction as previously described [14]. Briefly, 100 μl sample and 100 μl Griess reagent were added to a multiplate well and absorbance was read at 550 nm.

Arginase activity

Generation of ^{14}C-urea from ^{14}C-arginine in culture supernatants (extracellular arginase activity) was determined by the method of Russell and Ruegg [15]. After 48 hours of culture 150 μl of supernatant was removed and added to 0.8 ml of pH 4.5 buffer containing 250 mM acetic acid, 100 mM urea and 10 mM L-arginine (Sigma). Three hundred microliters of a continuously stirred suspension of Dowex resin (50W × 8,100 to 200 mesh H^+ form, 1 g/ml) was then added. The tubes were shaken for one minute and then centrifuged at 750 rpm (120G) for five minutes. Five hundred microliters of supernatant containing ^{14}C-urea was added to 3 ml Ecoscint scintillation fluid and the radioactivity was counted. Percentage conversion of ^{14}C-arginine to ^{14}C-urea was calculated as follows: [(cpm in 500 μl supernatant − cpm in medium without cells) / 0.36 × (cpm in 150 μl unseparated medium)] × 100.

Intracellular arginase activity of 10^6 thioglycollate macrophages was measured after 48 hours incubation. Medium was removed from each well and cells were lysed with 0.3 ml 0.01% Triton X-100 pH 7.5 containing bovine serum albumin (2 mg/ml), 10 mM $MnCl_2$ and 12 mM Na-maleate. Briefly, to 50 μl samples 150 μl of 100 mM glycine buffer pH 9.7 was added, samples and substrate were prewarmed and incubated in a waterbath at 37°C. Substrate of 50 μl 250 mM L-arginine containing L-[Guanido-^{14}C]-arginine (approximately 10^5 cpm/50 μml) was added. After exactly 20 minutes the reaction was stopped by adding 0.8 ml of pH 4.5 buffer containing 250 mM acetic acid, 100 mM urea and 10 mM L-arginine. ^{14}C-urea was separated using Dowex and counted on a scintillation counter as above. Units of intracellular arginase activity were calculated as follows: U/10^6 cells = μmol urea produced/min/10^6 cells at 37°C and pH 9.7 = 625 × [(cpm in 500 μl separated sample − cpm in separated medium without cells)/20 × cpm in 50 μl L-arginine substrate ^{14}C-arginine)].

Viability assay

Viability of isolated macrophages in culture after 48 hours was determined using a modified method described by Jones and Senft [16] using Fluorescein Diacetate (FDA, Sigma) 1 mg/ml in acetone and ethidium bromide (EI, Sigma) 5 mg/ml in RPMI stock solutions. A 50 μl drop of 2 ml PBS containing 20 μl of EI and 30 μl of FDA stock was placed on each well. The preparations were examined using a fluorescent microscope. With this method viable cells stain green while the nuclei of non-viable cells stain red. Viability was expressed as percentage viable/total cell counted, (mean total cells counted >300).

Protein assay

Protein content per well was estimated using a micro-BCA kit (Pierce; Rockford, Illinois, USA) after lysing macrophages with 1 ml 0.01% Triton X-100 and repeated freezing and thawing.

Statistics

All data are presented as mean ± SEM. NO_2^- production is expressed as nmol NO_2^-, extracellular arginase activity is expressed as % conversion of ^{14}C-arginine to ^{14}C-urea or as nmol urea calculated from % conversion and the known L-arginine concentration in the medium. Intracellular arginase activity is expressed as nmol urea produced per min at pH 9.7 and 37°C. Data were analyzed for statistical significance by paired or unpaired Student's *t*-test as appropriate with $P < 0.05$ taken as significant.

Results

Glomerulonephritis: NOS and arginase activity in glomeruli

Three days after i.v. nephrotoxic globulin nephritic rats were proteinuric (115 ± 16 mg/24 hrs, $N = 4$). Histology showed a mild proliferative glomerulonephritis.

Glomerular NO_2^- production is shown in Table 1. The basal NO_2^- production by nephritic glomeruli was higher than by control glomeruli. When stimulated with either 1 or 100 μg/ml LPS the NO_2^- production by nephritic glomeruli was significantly higher than when unstimulated or when compared to production by control glomeruli. LPS stimulated NO_2^- production in nephritic glomeruli was significantly inhibited by L-NMMA.

The metabolism of L-arginine to urea (extracellular arginase activity) by nephritic glomeruli is also shown in Table 1. For the

Table 1. NO_2^- production and arginase activity[a] of isolated glomeruli, (N = 4) after 48 hours in culture

		nmol NO_2^-/2000 glomeruli		nmol urea/2000 glomeruli	
	L-NMMA	Nephritic	Control	Nephritic	Control
Basal	−	7.4 ± 1.8	3.3 ± 0.2	84.3 ± 16[b]	14.8 ± 5.0
	+	4.8 ± 0.9	3.7 ± 0.6	99.3 ± 33	16.8 ± 2.3
LPS *1 μg/ml*	−	17.3 ± 3.6[b]	3.2 ± 1.0	63.6 ± 11[b]	16.0 ± 3.8
	+	5.0 ± 0.9[c]	2.8 ± 0.5	104.2 ± 29	19.3 ± 6.5
LPS *100 μg/ml*	−	61.8 ± 10.7[b,d]	4.8 ± 0.5	65.3 ± 12[b]	18.2 ± 3.6
	+	9.5 ± 2.2[c]	4.4 ± 1.8	159.0 ± 50	27.8 ± 9.0

[a] Expressed as nmol urea/2000 glomeruli
[b] $P < 0.033$ compared to control
[c] $P < 0.026$ compared to incubation without L-NMMA
[d] $P < 0.012$ compared to basal and LPS 1 μg/ml

first time we show that there is arginase activity in isolated glomeruli. Nephritic glomeruli showed a significant 5.7-fold increase ($P = 0.015$) in basal arginase activity (84.3 ± 16 nmol urea/2000 glom) compared with control glomeruli (14.8 ± 5 nmol urea/2000 glom).

When stimulated with 1 or 100 μg/ml LPS, arginase activity in nephritic glomeruli tended to decrease but was still significantly higher than in control glomeruli ($P \leq 0.022$). This effect of LPS was not observed in the control glomeruli. Addition of L-NMMA induced a 1.2- to 1.8-fold increase in arginase activity in nephritic glomeruli stimulated with 1 or 100 μg/ml LPS (104.2 ± 29 and 159 ± 50 nmol urea/2000 glom), although these changes did not reach statistical significance. In the control glomeruli addition of L-NMMA also induced a small, not significant increase in arginase activity. In controls, arginase activity never rose above 27.8 ± 9 nmol urea/2000 glom.

Calculated ratio's of arginase activity compared to NO_2^- production (A/N ratio) for each individual rat showed a predominance of the arginase pathway in nephritic and control glomeruli, both basally and stimulated. The A/N ratio was 4.8 to 1 in control glomeruli and in nephritic glomeruli this ratio was 14.2 to 1, almost threefold higher. After LPS stimulation the A/N ratio in nephritic glomeruli fell to 1.1 to 1 and in controls to 3.8 to 1. Addition of L-NMMA increased the A/N ratio in nephritic glomeruli both basally and when stimulated with LPS.

Peritoneal macrophages

Experiment 1: NOS and arginase activity in thioglycollate macrophages. NO_2^- production of thioglycollate (TG) macrophages after 48 hours in culture is shown in Figure 1. Stimulation with gamma-interferon (IFN) induced a significant increase in NO_2^- production, which could be significantly inhibited with L-NMMA. Highest NO_2^- production was seen after stimulation with 1 or 100 μg/ml LPS, also significantly inhibited with L-NMMA.

The metabolism of L-arginine to urea by peritoneal macrophages in culture (extracellular arginase activity) is shown in Figure 2. Basal extracellular arginase activity was present (7.8 ± 0.3 nmol urea/10^6 macrophages). Activity increased slightly after stimulation with IFN (10.9 ± 0.8) or LPS (12.8 ± 1.2 and 12.5 ± 0.6 nmol urea/10^6) but this increase did not reach statistical significance. However, when L-NMMA was added, basal and IFN or LPS stimulated macrophages showed a fourfold, significant increase ($P \leq 0.002$) in arginase activity.

Ratios of extracellular arginase activity compared to NO_2^- production (A/N ratio) of macrophages showed a predominance of the NOS pathway, which reversed to arginase predominance after addition of L-NMMA. The basal A/N ratio was 0.28 to 1 and fell to 0.20 to 1, and 0.16 and 0.15 to 1 after stimulation with IFN, LPS 1 or 100 μg/ml, respectively. Addition of L-NMMA increased the A/N ratio both basally and when stimulated with either IFN or LPS to around 3 to 1.

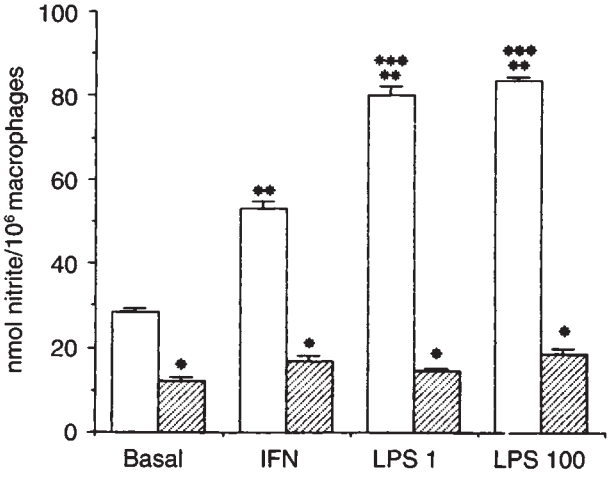

Fig. 1. *Nitrite production by thioglycollate macrophages after 48 hours in culture.* Hatched columns represent incubations with 300 μM L-NMMA, $N = 6$ except LPS 100 ($N = 3$). *$P < 0.014$ compared to incubations without LNMMA; **$P < 0.001$ compared to basal; ***$P < 0.001$ compared to IFN.

Figure 3 shows the intracellular arginase activity of TG macrophages lysed after 48 hours in culture with or without IFN or LPS. The basal intracellular arginase activity was 19.3 nmol urea/min/10^6 macrophages. Activity significantly increased ($P \leq 0.002$) after stimulation with LPS (71.3 ± 4.7) or IFN (39 ± 2.2 nmol urea/min/10^6 macrophages).

Experiment 2: Comparison of NO_2^-, arginase and viability in thioglycollate and C. parvum macrophages. Effects of low L-arginine concentrations on NO_2^- production. We compared thioglycollate (TG) elicited and C. parvum activated macrophages and investigated the effects of arginine depletion on NOS through incubation in L-arginine free (LAF) medium.

Table 2 compares L-arginine metabolism in TG and C. parvum macrophages, and therefore compares the difference between elicited and activated macrophage types. Basal NO_2^-

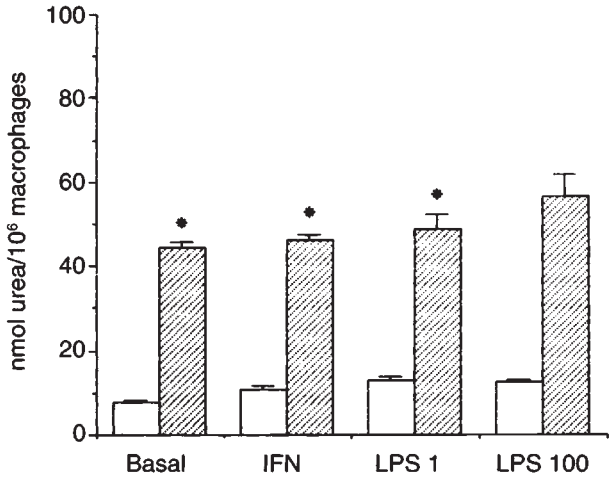

Fig. 2. *Extracellular arginase activity of thioglycollate macrophages after 48 hours in culture, calculated from conversion of ^{14}C-arginine to ^{14}C urea.* Hatched columns represent incubations with 300 μM L-NMMA; $N = 6$, except LPS 100 ($N = 3$). *$P \leq 0.002$ compared to incubations without LNMMA.

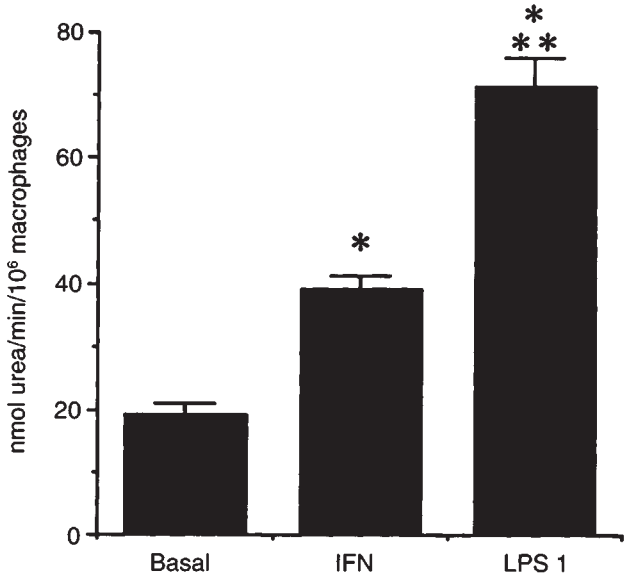

Fig. 3. *Intracellular arginase activity of thioglycollate macrophages after 48 hours in culture,* N = *3.* *$P = 0.002$ compared to basal; **$P = 0.008$ compared to IFN.

production was significantly higher in C. parvum than TG macrophages, and was significantly inhibited with L-NMMA in both. Gamma-IFN stimulation increased NO_2^- production in TG macrophages, but not in C. parvum macrophages. Extracellular arginase activity in contrast was lower in C. parvum macrophages. As before, after addition of L-NMMA the arginase activity increased significantly both in basal and stimulated TG and C. parvum macrophages.

In the additional experiments where LAF medium was used basal and stimulated NO_2^- production by macrophages was significantly reduced: basal 15.1 ± 1.5 (TG), 50.7 ± 0.6 (C. parvum), $P \leq 0.02$; gamma-IFN 21.4 ± 1.5 (TG), 40.3 ± 0.2 (C. parvum), $P \leq 0.02$ compared with results in normal medium.

In both TG and C. parvum macrophages the basal viability of 92 and 85% fell after stimulation with IFN to 75 and 73% respectively, an effect which was abolished by addition of L-NMMA or incubation in LAF medium.

Figure 4 shows the intracellular arginase activity of C. parvum macrophages lysed directly after harvest and adherance or after 48 hours in culture with or without IFN. Intracellular activity directly after harvest and adherance was very low, 0.72 ± 0.2 nmol urea/min/10^6 macrophages. Basal intracellular arginase activity after 48 hours was 6.5 ± 0.7 nmol urea/min/10^6 macrophages. Unlike in TG macrophages (Fig. 3), this activity could not be significantly stimulated with IFN. The intracellular arginase activity in lysed C. parvum macrophages was half that of lysed TG macrophages.

Discussion

In this study we show activity of both NO synthase and arginase pathways of L-arginine metabolism in glomeruli. We have previously demonstrated that nephritic glomeruli metabolise arginine to nitric oxide [1, 2], and have shown that infiltrating macrophages are the major source [3]. Arginine may also be metabolized to urea and ornithine, and this reaction is catalyzed by arginase.

In the present study we demonstrate that glomeruli in culture possess arginase activity, that this activity is at least six times higher in nephritic glomeruli compared with controls. It appears to account for most of the arginine metabolism under basal conditions. In RG immunized control glomeruli, although the absolute values are lower than in nephritic glomeruli, the arginase pathway is also the main pathway of arginine metabolism.

It is important to consider the source of this arginase activity in both nephritic and control glomeruli. Arginase activity has been found in various cell types including fibroblasts [17, 18], and macrophages [5–8, 11, 12, 15, 19, 20], and Albina et al [7] have suggested that in wounds macrophages are likely to be the major source of arginase. In the glomerulus the possible sources are macrophages or intrinsic glomerular cells. We therefore studied the levels of arginase activity in both elicited and activated types of peritoneal macrophages under various conditions.

The results we found for macrophage arginase activity are comparable with those in other studies [8, 11, 20]. The maximal arginase activity (without L-NMMA) we found was in thioglycollate elicited macrophages stimulated with LPS; these cells produced 12.8 nmol urea/10^6 cells/48 hours. This compares with production by nephritic glomeruli of 84.3 nmol/2000 gloms/48 hours. We have found previously that macrophage infiltration in this model amounts to a maximum of 200 macrophages/glomerulus and thus 2000 glomeruli might maximally contain 0.4×10^6 macrophages. From these figures infiltrating macrophages are unlikely to be the source of the glomerular arginase activity we have measured. Further supporting this, macrophages infiltrating glomeruli in immune complex glomerulonephritis are of activated type, resembling C. parvum cells rather than thioglycollate elicited macrophages [21, 22]. In our experiments C. parvum cells showed less arginase activity than those elicited by thioglycollate. In addition, in unstimulated nephritic glomeruli the arginase pathway predominates,

Table 2. Nitrite production, extracellular arginase activity[d] and viability of thioglycollate (TG) and C. parvum (CP) macrophages ($N = 3$)

	nmol $NO_2^-/10^6$		% Conversion/10^6		% Viable	
	TG	CP	TG	CP	TG	CP
Basal	28.3 ± 2.1	61.9 ± 0.6[c]	1.6 ± 0.3	1.1 ± 0.3	92	85
L-NMMA	0.9 ± 0.2[a]	13.8 ± 3.4[a]	4.7 ± 0.2[a]	1.8 ± 0.4[c]	95	91
Gamma IFN	55.1 ± 2.0[b]	56.9 ± 0.5[b]	2.3 ± 0.2	1.5 ± 1.0	75	73
Gamma IFN/L-NMMA	4.6 ± 0.4[a]	17.4 ± 4.7	4.3 ± 0.1[a]	3.8 ± 0.2	94	94

[a] $P < 0.045$ compared to incubation without L-NMMA
[b] $P < 0.022$ compared to basal
[c] $P < 0.025$ compared to TG
[d] Expressed as % conversion of ^{14}C L-arginine to ^{14}C urea

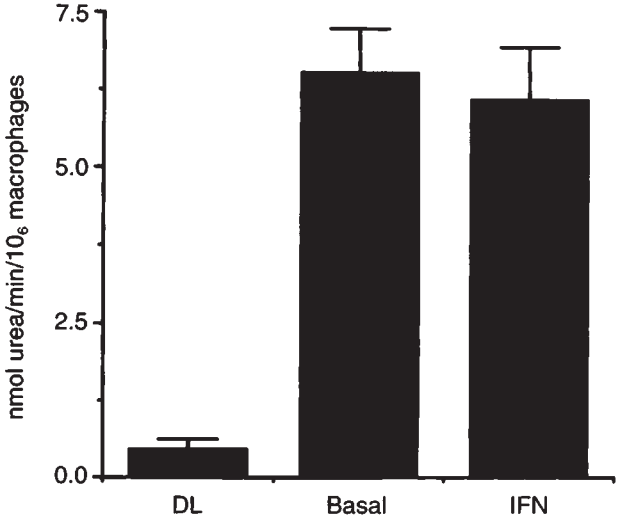

Fig. 4. *Intracellular arginase activity of C. parvum macrophages, directly lysed after harvest and incubation (DL) or after 48 hours in culture (basal, IFN)* N = 3.

whereas in thioglycollate macrophages the ratio of urea/nitrite production was 0.28 and in C. parvum macrophages even lower, 0.07. We therefore suggest that much of the arginase activity may originate from intrinsic glomerular cells rather than macrophages, although a definite answer will require isolation of cell populations from nephritic glomeruli.

It has been suggested that NO produced by macrophages in culture induces cell death. We have shown that in macrophages there is a loss of viability after IFN stimulation, an effect which is abolished by the NOS inhibitor L-NMMA and reduced in L-arginine free medium. These results also suggest, as has been previously reported [23], that intracellular sources of L-arginine exist.

The relation between intra- and extracellular arginase activity has not yet been fully established. We showed higher intracellular arginase activities in TG macrophages than C. parvum macrophages which corresponded with their extracellular arginase activity. Intracellular activity could be stimulated by IFN or LPS in TG macrophages, which was not reflected in the extracellular activity possibly because of the fact that the increased activity of NOS competes for substrate.

The presence of the arginase pathway within glomeruli raises the possibility that macrophages and intrinsic glomerular cells may compete for arginine and that increased glomerular arginase activity in glomerulonephritis might limit the arginine available for NO production. If this were so, then an increase in urea generation should occur if the NOS pathway is inhibited. Indeed, we did find increased urea from nephritic glomeruli treated with L-NMMA, but this did not reach statistical significance. We found a decrease in urea production by nephritic glomeruli when NOS was stimulated with LPS, which also suggests competition. The activity of each pathway may vary with time as has been shown for wounds [7], and therefore sequential studies will be of interest.

Apart from possible competition for arginine, arginase in glomeruli may also play a role in mesangial proliferation and glomerular scarring. Arginine metabolism via arginase produces L-ornithine which is a precursor for proline, which can be incorporated into collagen, and also for polyamines. An increase in generation of polyamines has been shown to be associated with proliferation and repair in various cell types. Recently Schulze-Lohoff et al [24] have shown that ornithine decaboxylation to polyamines is associated with mesangial cell proliferation.

In conclusion, we have demonstrated that glomeruli in vitro are able to metabolize arginine via the arginase pathway as well as via the nitric oxide synthase pathway. Arginase activity is present at a low level in control glomeruli predominant over nitrite production through NOS, and is markedly increased in nephritic glomeruli. The data from peritoneal macrophages suggest that infiltrating macrophages are unlikely to be the major source of arginase in nephritic glomeruli. The presence of both pathways of arginine metabolism in glomeruli implies possible competition for available substrate, and a balance between the haemodynamic and cytotoxic effects of NO and the stimulatory effect of products of the arginase pathway on cell proliferation and matrix production. Glomerular arginase activity may therefore have major implications for glomerular pathophysiology

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